

Botrocetin (venom coagglutinin): Reaction with a broad spectrum of multimeric forms of factor VIII macromolecular complex

(platelet aggregation/von Willebrand factor/ristocetin testing)

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ABSTRACT Botrocetin, originally called venom coagglutinin, is a *Bothrops* factor that causes aggregation of blood platelets in the presence of the von Willebrand component of the factor VIII macromolecular complex. The complex consists of a series of multimers with a molecular weight of about $1-20 \times 10^6$. Ristocetin, another agent that causes platelet aggregation dependent on von Willebrand factor, reacts with only the higher molecular weight multimers. We report on the reactivity of botrocetin in relation to the multimeric structure of the factor VIII complex. Several plasmas or plasma fractions with abnormal distribution of the multimeric sizes were examined, including variant von Willebrand disease type IIA with lack of the higher molecular weight forms, commercial antihemophilic factor concentrates with a preponderance of lower molecular weight forms, cryoprecipitate-free plasma containing mainly the smaller multimers, and a chromatographic fraction of plasma containing only the highest molecular weight polymers. Factor VIII-related antigen content was adjusted to 25-100%. All of the preparations lacking the high molecular weight forms caused prompt platelet aggregation with botrocetin, but none of them caused aggregation in the ristocetin test made isochronal with the botrocetin test. The very high molecular weight polymers were equally effective with botrocetin and ristocetin. These findings indicate that the *Bothrops* factor is reactive with a broad spectrum of high to low molecular weight forms of the factor VIII complex, suggesting that bioassays of von Willebrand factor with botrocetin should correlate better with immunoassays for factor VIII-related antigen and could reflect better the full platelet-aggregating function of the complex than do ristocetin determinations.

A factor found in the venom of several snakes, mainly in the *Bothrops* species, has been shown to be a unique "activator" of the platelet aggregation reaction that is dependent on the von Willebrand factor (vWF). vWF, named because of its absence in the severe form of the inherited bleeding disorder "designated von Willebrand disease (vWD)," is part of the factor VIII macromolecular complex. The complex possesses two types of biological activities. One is the antihemophilic factor (AHF) or coagulant factor VIII (factor VIII:C) activity which corrects the bleeding defect in hemophilia A. The other type is vWF activities (factor VIIIIR:vWF) which correct the platelet-related functional defect in vWD. These two types of activities of the complex can be measured immunologically and are designated as the factor VIII coagulant antigen (factor VIII:CAG) and the factor VIII-related antigen (factor VIIIIR:Ag), respectively. The complex in normal plasma consists of a series of macromolecular polymers of molecular weights ranging from about 10^6 to 20×10^6 .

The uniqueness of the venom factor, originally called "venom coagglutinin (VCA)" and more recently called "botrocetin," is

that at present it is the only material that can be used to identify and quantify the platelet-aggregating vWF regardless of its mammalian source (1, 2). Both ristocetin, the other known type of activator of vWF-dependent platelet aggregation, and botrocetin can be used to measure vWF activity in human plasma (3), but vWF in most mammalian plasmas is resistant to ristocetin (2, 4). Ristocetin is known to cause platelet aggregation with only the larger polymers or multimers of the factor VIII macromolecular complex (5-9). This has led to the concept that the platelet-aggregating function of the complex resides primarily in the high molecular weight multimers of factor VIIIIR:Ag. Thus, plasmas from certain of the variants of vWD, known as type IIA (6, 7, 10-12) which lack the highest molecular weight multimers, do not aggregate platelets with ristocetin yet have nearly normal or higher than normal levels of factor VIIIIR:Ag. Furthermore, the supernatant plasma remaining after the removal of cryoprecipitate contains mainly the lower molecular weight multimers of factor VIIIIR:Ag and does not aggregate platelets in the presence of ristocetin (7, 10, 12, 13).

The above observations led us to study the reactivity of botrocetin with a number of plasmas or plasma preparations with an abnormal distribution of factor VIIIIR:Ag multimers to determine if the pattern of reactivity of botrocetin was restricted to the high molecular weight multimers as with ristocetin or if it reacted with a broader spectrum of the polymers of the factor VIII complex. The factor VIII macromolecular preparations studied contained either (i) a predominance of the lower molecular weight multimers with marked reduction or absence of the higher molecular weight forms or (ii) only the highest molecular weight polymers with absence of the smaller multimeric types. It was found that botrocetin induced platelet aggregation with all of the preparations tested, including those that were inactive with ristocetin. Thus, botrocetin is also unique in respect to its platelet-aggregating ability with the whole spectrum of factor VIIIIR:Ag multimers.

MATERIALS AND METHODS

Materials. Ristocetin (Lundberg, Copenhagen) was dissolved in isotonic saline at the desired concentration. Botrocetin was prepared from the venom of *Bothrops jararaca* as described (1) and was assayed for potency in units per ml (14). One unit of botrocetin is defined as that amount in 1 ml that causes aggregation of lyophilized platelets in 14 sec in a standardized procedure (14). The stock solution of botrocetin (500 units/ml) was diluted with imidazole-buffered saline. Lyophilized human platelets (15) were made from a blood bank platelet concentrate 72 hr old (16) with fixation in paraformaldehyde (17). The rabbit

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Abbreviations: vWF, von Willebrand factor; vWD, von Willebrand disease; AHF, antihemophilic factor; factor VIII:C, coagulant factor VIII; factor VIIIIR:Ag, factor VIII-related antigen; factor VIII:CAG, factor VIII coagulant antigen.

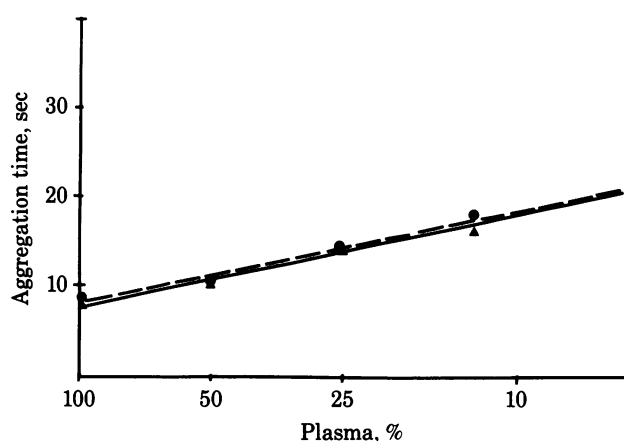


FIG. 1. Analysis of vWF activity by using isochronal platelet-aggregation tests with botrocetin and with ristocetin. Equilibration was obtained by adjusting the final concentration of botrocetin (12.5 units/ml) and ristocetin (0.5 mg/ml) to give an aggregation time of 8 sec with undiluted reference plasma.

antiserum to human factor VIII:Ag and its preparation have been described (12). Cryosupernatant was cryoprecipitate-free human plasma (16). Cryoprecipitate (16) was obtained from American Red Cross Blood Services (Columbia, SC). Factor VIII concentrate [antihemophilic factor (human), Hemofil, Hyland, Costa Mesa, CA) was dissolved in 20 ml of distilled water and then diluted with imidazole-buffered saline. Type IIA vWD plasmas were from two subjects, V.N., a 26-year-old black female, and W.R., a 17-year-old black male (12). Human reference plasma was a pool of platelet-poor plasmas from six healthy subjects, ages 20–30 years, who denied ingestion of drugs during the two weeks preceding blood sampling.

Methods. Platelet-aggregating tests for vWF with ristocetin and with botrocetin (VCA) have been described (2, 3). Both the macroscopic tap-tube and the aggregometry methods were used. For macroscopic testing of cryosupernatant, platelets were suspended in cryosupernatant (3 vol) before addition of activator (1 vol). A modified Laurell method for determining factor VIII:Ag was that described by Lamb *et al.* (12), as was the two-dimensional electrophoresis using 1% agarose gel.

RESULTS

Equilibration of Ristocetin and Botrocetin Testing of vWF. The rate of platelet aggregation induced with either ristocetin

or botrocetin was used to measure the expression of vWF activity. It is known that the rate of platelet aggregation is dependent not only on the concentration of vWF in the test material but also on the concentration of botrocetin or ristocetin (3). The normal reference plasma by definition had 100% factor VIII:Ag with a full range of multimer sizes and 100% vWF activity. The concentrations of botrocetin and ristocetin were standardized so that the rate of platelet aggregation was the same with both activators and human reference plasma. An 8-sec aggregation time was selected for each activator with undiluted plasma. It will be noted in Fig. 1 that human reference plasma serially diluted caused platelet aggregation at nearly the same rate with the adjusted concentrations of ristocetin and botrocetin (Fig. 1). This permitted reliable assessment of the comparability of response of test samples having an abnormal distribution of factor VIII:Ag multimers.

Botrocetin Reactivity with Lower Molecular Weight Multimeric Forms of Factor VIII:Ag. Table 1 lists the plasmas and plasma fractions that were studied along with data from a representative analysis of each the various test and control samples. Each test sample was adjusted, as needed, to a factor VIII:Ag level in the range 25–100% and was compared with normal human reference plasma. Data on the parameters of migration of VIII:Ag in two-dimensional crossed immunoelectrophoresis are given. The maximum lateral migration was comparable for all samples, both test and control. However, all of the test samples showed the beginning of the immunoprecipitin arc at a considerable interval (4.0–6.4 mm) from the well (these intervals are indicated as gap distances). The location of the peak of the arc in relation to the lateral migration was anodal to the peak for normal plasma. These data indicate a decrease in the highest molecular weight multimers and a predominance of smaller forms in all of the test samples.

The plasma from the two patients with type IIA vWD previously had been documented as lacking the higher molecular weight multimers but with factor VIII:Ag levels in the range 160–470% (W.R.) and 44–61% (V.N.) (12). The plasmas from both patients failed to induce platelet aggregation with ristocetin. On the other hand, aggregation was rapid with botrocetin, indicating that it is reactive with the lower molecular weight multimers of factor VIII:Ag.

The commercial factor VIII concentrate of normal human plasma lacked the highest molecular weight multimers (Table 1). After dilution of the reconstituted concentrate sufficiently to give a factor VIII:Ag value equivalent to that of normal plasma, the macroscopic ristocetin test was negative. Neverthe-

Table 1. Reactivities of lower molecular weight multimers of factor VIII:Ag with ristocetin and botrocetin

Plasma or plasma fractions	Factor VIII:Ag, %	Two-dimensional crossed immunoelectrophoresis migration distance, mm			Macroscopic vWF test, sec		Multimeric distribution of factor VIII:Ag	
		Lateral	Gap	Peak	Ristocetin	Botrocetin	Higher	Lower
							<i>M_r</i>	<i>M_r</i>
vWD plasmas, type IIA:								
Subject W.R.	43	15	4.6	8.6	Negative	14	—	+
Subject V.N.	78	13.1	5.5	8.5	Negative	14	—	+
Plasma fractions:								
Factor VIII concentrate*	100	12.4	6.4	9.6	Negative	18	Trace	+
Cryosupernate†	25	12.5	4.0	8.2	Negative	30	—	+
Human reference plasma:								
Undiluted	100	13.8	0	6.1	8	8	+	+
Diluted 1:4	25	12.6	2.0	6.3	14	14	+	+

* Sample for vWF test had 25% factor VIII:Ag.

† Sample for vWF test had 100% factor VIII:Ag.

less, the diluted concentrate caused rapid aggregation of platelets with botrocetin. Disparate results were also obtained with the aggregometric method with botrocetin and ristocetin (Fig. 2). These data indicate that botrocetin is reactive with the lower molecular weight multimers of factor VIII:Ag. The lack of a sufficient concentration of higher molecular weight multimers in this commercial concentrate appears to be responsible for the negative ristocetin test because both the ristocetin and botrocetin tests were positive with the undiluted preparation which had a factor VIII:Ag value $>1,000\%$.

The cryosupernatant plasma fraction was used as a source of the lowest molecular weight multimers of VIII:Ag. The ristocetin test was negative (Table 1). These plasma fractions caused prompt vWF-dependent platelet aggregation with botrocetin. This study indicates that botrocetin is reactive with the lowest molecular weight multimers of the factor VIII:Ag.

Botrocetin Reactivity with Higher Molecular Weight Multimeric Forms of Factor VIII:Ag. The source of the largest multimers of factor VIII:Ag was the first eluting fractions of cryoprecipitate in the void volume upon Sepharose 4B chromatography (Table 2). The first three fractions with activity (fractions 34–36) were nearly equally active with ristocetin and botrocetin. The protein content was low as judged by optical density at 280 nm. These data indicate that botrocetin detects the first eluted and largest multimers of factor VIII:Ag obtained by chromatography.

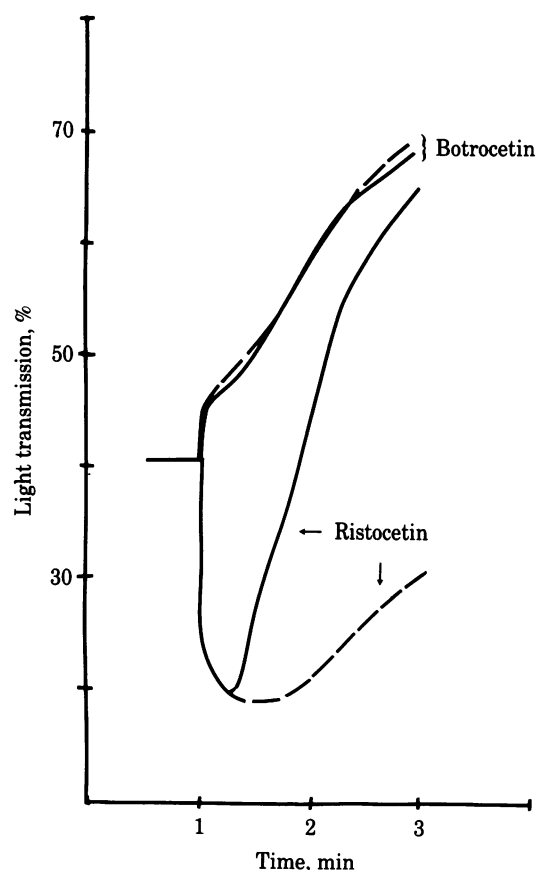


FIG. 2. Aggregometric study of responses of factor VIII concentrate (---) with ristocetin and botrocetin, compared with responses of normal reference plasma (—). Test mixtures: for ristocetin testing, 0.18 ml of 0.084 M imidazole-buffered saline (pH 7.35), 0.1 ml of plasma (1:4) or concentrate (25% factor VIII:Ag), 0.1 ml of lyophilized platelets ($800,000/\text{mm}^3$), and 0.02 ml of ristocetin (20 mg/ml); for botrocetin testing, 0.19 ml of buffer, 0.1 ml of plasma or concentrate, 0.1 ml of platelets, and 0.01 ml of botrocetin (500 units/ml).

Table 2. Reactivity of vWF is similar with ristocetin and botrocetin in chromatographic fractions containing highest molecular weight multimers

Fraction* no.	Absorbance at 280 nm	Macroscopic vWF test, sec		Factor VIII:Ag, %
		Ristocetin	Botrocetin	
32	0.0	60	60	Neg.
33	0.0	60	60	Neg.
34	0.005	34	28	Neg.
35	0.015	7.3	7.0	44
36	0.025	5.0	5.0	143
40–50	0.0625–0.075	4–6	4–6	181–194

* A 5.0-ml sample of cryoprecipitate was chromatographed on a Sepharose 4B (Pharmacia) column, 1.6×70 cm. Elution buffer was 0.05 M Tris-buffered saline containing 5 mM trisodium citrate (pH 7.35). The collected sample was 0.8 ml per tube.

DISCUSSION

Botrocetin, originally called venom coagglutinin (1), was discovered several years ago during a search for an “activator” of the vWF–platelet reaction that did not have the limitations of the only known activator at that time, ristocetin. Ristocetin is a protein-precipitating reagent and thus can cause false-positive reactions in aggregometric testing. Also, ristocetin is highly restrictive in its reaction with the plasma vWF: it induces little or no platelet aggregation with most mammalian plasmas and is unreactive with most species of platelets except for those of human origin (2, 4). Also, ristocetin has only limited reactivity with human factor VIII:Ag, being restricted to the high molecular weight multimers (5–9). This study was designed to examine the reactivity of botrocetin with the full range of multimers of human factor VIII:Ag in causing platelet aggregation and to compare the action of botrocetin with that of ristocetin.

The first set of experiments was designed to test the platelet-aggregating effect of botrocetin with factor VIII:Ag preparations that were unreactive with ristocetin and were largely lacking in the higher molecular weight forms. Three preparations were tested: variant vWD type IIA plasma from two patients, a high-purity commercial preparation of factor VIII (AHF), and the supernatant plasma remaining after preparation of cryoprecipitate. To obtain comparability of response with the two “activators,” all preparations were adjusted to a factor VIII:Ag level within the range 25–100% of normal reference plasma. Also, the concentrations of ristocetin and botrocetin were adjusted in the macroscopic procedure for determining vWF so that equivalent platelet aggregation times were obtained with each reagent and normal reference plasma (Table 1). This latter control procedure is believed to be needed because the rate of platelet aggregation in such tests is dependent not only on the amount of vWF but also on the amount of the activator (3).

Two-dimensional crossed immunoelectrophoresis demonstrated the lack of the high molecular weight multimers in the plasmas of two subjects with variant vWD. This is a confirmation of similar data on these two plasmas previously reported (12). These plasmas were inactive in the ristocetin screening test for vWF. Thus, they lacked the so-called ristocetin cofactor. However, both plasmas caused prompt platelet aggregation with botrocetin. These findings suggest that botrocetin testing would be a valuable additional diagnostic procedure for detecting type IIA vWD.

Although extremely rich in factor VIII:C activity, the commercial factor VIII concentrate has a greatly reduced content of the higher molecular weight multimers of factor VIII:Ag (Table 1) (12, 18) and is relatively ineffective in correcting the prolonged bleeding time in severe vWD (19). When the concen-

trate was sufficiently diluted, it caused no platelet aggregation with ristocetin, a finding in keeping with the depletion of high molecular weight multimers. Platelet aggregation was prompt with botrocetin.

The last of the three preparations with depleted high molecular weight multimers of factor VIII:Ag was the cryosupernatant harvested in the preparation of therapeutic cryoprecipitate concentrates of AHF (factor VIII:C). The smaller molecular weight multimers mainly go into solution when frozen plasma is thawed at 4°C, but only limited amounts of larger molecular weight multimers are solubilized. The ristocetin test was negative, as expected, but the botrocetin test resulted in platelet aggregation. The above group of experiments demonstrate that the lower molecular weight multimers of factor VIII:Ag are capable of causing prompt platelet aggregation with botrocetin.

The last experiment in this study was designed to test whether botrocetin was reactive with the highest molecular weight multimers of factor VIII:Ag, as is ristocetin. It has been demonstrated by Sepharose column chromatography of cryoprecipitate that the first eluates contain only the highest molecular weight multimers (8, 20). We took advantage of this phenomenon and tested the early elution fractions for platelet-aggregating vWF with both ristocetin and botrocetin (Table 2). The fractions caused prompt platelet aggregation with both reagents, and aggregation times were approximately the same in the two tests. In the first fraction, the botrocetin-induced aggregation time was somewhat shorter than that with ristocetin. Thus, it is concluded that botrocetin is reactive with the highest molecular weight multimers of factor VIII:Ag.

In spite of the heterogeneity of factor VIII:Ag in plasma and plasma fractions as well as the lack of reactivity of the mass of smaller macromolecules with ristocetin, botrocetin was found to be effective with the full range of sizes of the factor VIII:Ag multimers in inducing platelet aggregation. This implies that a correlation between vWF bioassay values with botrocetin and immunoelectrophoretic determination of factor VIII:Ag should be better than is the case for ristocetin assays. "Ristocetin cofactor" is a term frequently used for the high molecular weight multimers of factor VIII:Ag that cause platelet aggregation with ristocetin. If one wanted an analogous term for the multimers that cause platelet aggregation with botrocetin it would be "botrocetin cofactor." Botrocetin cofactor values could be a better indicator of the total potential platelet-aggregating activity of plasma vWF than are ristocetin cofactor values.

Note Added in Proof. Since this manuscript was submitted, Howard *et al.* (21) have published data on variant vWD similar to those shown in Table 1.

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1. Read, M. S., Shermer, R. W. & Brinkhous, K. M. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 4514–4518.
2. Read, M. S., Potter, J. Y. & Brinkhous, K. M. (1983) *J. Lab. Clin. Med.* **101**, 74–82.
3. Brinkhous, K. M. & Read, M. S. (1980) *Blood*, **55**, 517–520.
4. Brinkhous, K. M., Thomas, B. D., Ibrahim, S. A. & Read, M. S. (1977) *Thromb. Res.* **11**, 345–355.
5. Over, J., Bouma, B. N., van Mourik, J. A., Sixma, J. J., Vlooswijk, R. & Bakker-Woudenberg, I. (1978) *J. Lab. Clin. Med.* **91**, 32–46.
6. Meyer, D., Obert, B., Pietu, G., Laverigne, J. M. & Zimmerman, T. S. (1980) *J. Lab. Clin. Med.* **95**, 590–602.
7. Pietu, G., Obert, B., Larrieu, M. J. & Meyer, D. (1980) *Thromb. Res.* **19**, 671–685.
8. Perret, B. A., Furlan, M., Kneubuehl, F. & Beck, E. A. (1981) *Biochim. Biophys. Acta* **669**, 98–104.
9. Gralnick, H. R., Williams, S. B. & Morisato, D. K. (1981) *Blood* **58**, 387–397.
10. Ruggeri, Z. M. & Zimmerman, T. S. (1980) *J. Clin. Invest.* **65**, 1318–1325.
11. Ruggeri, Z. M., Pareti, F. I., Mannucci, P. M., Ciavarella, N. & Zimmerman, T. S. (1980) *N. Engl. J. Med.* **302**, 1047–1051.
12. Lamb, M. A., Reisner, H. M., Cooper, H. A. & Wagner, R. H. (1981) *J. Lab. Clin. Med.* **98**, 751–763.
13. Doucet-de Bruine, M. H. M., Sixma, J. J., Over, J. & Beeser-Visser, N. H. (1978) *J. Lab. Clin. Med.* **92**, 96–107.
14. Brinkhous, K. M., Barnes, D. S., Potter, J. Y. & Read, M. S. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 3230–3234.
15. Brinkhous, K. M. & Read, M. S. (1978) *Thromb. Res.* **13**, 591–597.
16. Widmann, F. K., ed. (1981) *Technical Manual of the American Association of Blood Banks* (Washington, DC), 8th Ed., pp. 38–49.
17. Allain, J. P., Cooper, H. A., Wagner, R. H. & Brinkhous, K. M. (1975) *J. Lab. Clin. Med.* **85**, 318–328.
18. Weinstein, M. & Deykin, D. (1979) *Blood* **53**, 1095–1105.
19. Blatt, P. M., Brinkhous, K. M., Culp, H. R., Krauss, J. S. & Roberts, H. R. (1976) *J. Am. Med. Assoc.* **236**, 2770–2772.
20. Martin, S. E., Marder, V. J., Francis, C. W. & Barlow, G. H. (1981) *Blood* **57**, 313–323.
21. Howard, M. A., Salem, H. H., Thomas, K. B., Hau, L., Perkin, J., Coghlan, M. & Firken, B. G. (1982) *Blood* **60**, 1420–1428.